

# Cytotoxic and Mutagenic Effects of Specific Carcinogen-DNA Adducts in Diploid Human Fibroblasts

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A comparison of the cytotoxicity and mutagenicity of a series of carcinogens in normal diploid human fibroblasts and in cells deficient in one or more DNA repair processes has provided insight into the specific DNA adduct(s) responsible for these biological effects. The carcinogens tested include ultraviolet radiation; reactive derivatives of structurally related aromatic amides; metabolites of benzo(a)pyrene; the simple alkylating agents *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-ethyl-*N*-nitrosourea; and aflatoxin B<sub>1</sub> dichloride, a model for the reactive 2,3-epoxide of aflatoxin B<sub>1</sub>. Exponentially growing cells were exposed to agents and assayed for mutations (induction of 6-thioguanine resistance) and cell killing (loss of colony-forming ability). Cells deficient in repair of particular DNA adducts or lesions proved more sensitive to the agent causing those lesions than did normally repairing cells. Many of the carcinogens were compared for their mutagenic and/or cytotoxic effect, not only as a function of dose administered, but also as a function of the initial number of adducts or photoproducts induced in DNA and the number remaining at critical times posttreatment. Density-inhibited cultures were exposed to cytotoxic and mutagenic doses. Immediately after treatment, or after various lengths of time in confluence, the cells were harvested and analyzed for the number of lesions remaining in DNA. A portion was plated at lower densities and assayed for mutations and/or for survival. In several instances, the adducts were analyzed by high-pressure liquid chromatography. As an alternative approach, cells were synchronized and treated at various times prior to the onset of DNA synthesis and analyzed for survival and/or the frequency of mutations. The results demonstrated a high correlation between the number of DNA lesions remaining unexcised at the time the DNA was replicated and the frequency of mutations induced. Comparative studies of the frequency of UV-induced transformation (to anchorage independence) of normal and repair-deficient cells showed this also to be true for transformation.

## Introduction

A central question under investigation in many laboratories concerns the biological effects of DNA adducts and photoproducts induced in cells by exposure to environmental mutagens and carcinogens. As part of ongoing studies of the mechanisms of carcinogenesis, we and our co-workers have investigated the cytotoxicity, mutagenicity, and transforming activity of a number of chemical carcinogens and radiation in diploid human fibroblasts in culture. In particular, we have made use of human cells which differ in their ability to excise or remove such lesions from their DNA to determine the answer to the following. Is cell killing correlated with the initial number of DNA adducts or the number of remaining at a particular time posttreatment? Are potentially mutagenic lesions converted into mutations

during excision repair? What is the role of semiconservative DNA replication in mutation induction? Are particular lesions or adducts intrinsically more mutagenic or more cytotoxic than others? Are genetic changes resulting from unrepaired DNA lesions causally involved in the process of neoplastic transformation?

To address these questions, we developed quantitative assays for measuring the cytotoxic, mutagenic, and oncogenic effect of carcinogens in diploid human skin fibroblasts derived from neonatal foreskin or skin biopsy material (1-8). The use of human cells offers a number of advantages. They grow well in culture and clone with 40 to 80% efficiency. They can be treated with direct-acting mutagens, and the cytotoxic effect quantitated from the decrease in their cloning ability. Compounds requiring activation can also be used if the cells are provided with a source of activation, such as metabolizing feeder cells (9,10). The dose-dependent induction of mutations by carcinogens can be quantitated by determining the frequency of cells able to grow in the

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presence of the base analogs 8-azaguanine or 6-thioguanine (TG) as a result of inactivation of a functional hypoxanthine(guanine)phosphoribosyltransferase (HPRT) (2,11). Another genetic marker we have used to measure mutation induction is resistance to diphtheria toxin (DT) which results from inactivation of its receptor site on the elongation factor 2 used in protein synthesis (12). The frequency of transformation of normal human cells can be measured by determining the frequency of cells able to exhibit anchorage-independent growth, i.e., colony formation in semisolid medium containing 0.33% agar (5,8). We and others have shown that human cells isolated from anchorage-independent colonies can produce nodular growths when propagated and injected into X-irradiated athymic mice, whereas cells that do not express this phenotype do not grow in these animals (5,8,13,14).

Although strains of human fibroblasts in culture do not have an infinite life span (15), the number of cells one can obtain from a single fibroblast population is so large (16) that it provides enough cells for many workers for several years. The advantage of using these human fibroblast cell lines over the commonly used rodent cell lines with infinite life spans is that the former retain their diploid karyotype throughout their life span, whereas the latter usually are aneuploid (17) and therefore no longer faithfully represent the normal cells of the species from which they were obtained.

Another advantage of working with strains of diploid human fibroblasts is that they retain the excision repair capability present in the individual from which they were obtained, whereas cell lines, especially those of rodent origin, often lose their characteristic excision repair capabilities with time in culture (18). Most importantly, human fibroblast cell strains deficient in rate of DNA repair are available, e.g., from xeroderma pigmentosum (XP) patients (19) or other sources (20). Cells from the majority of XP patients have a reduced rate of excision of pyrimidine dimers (21) or adducts formed by the covalent linkage of bulky ring structures to DNA, such as those formed by reactive derivatives of aromatic amides (1,22,23), polycyclic aromatic hydrocarbons (11,24,25), or aflatoxins (26,27). They are normal in their ability to repair lesions induced by methylating agents, such as MNNG (28). Recently, human cell lines deficient in O<sup>6</sup>-methylguanine-DNA-methyltransferase (MT) activity have been identified (20). These repair-deficient lines are very useful for determining the biological significance of O<sup>6</sup>-methylguanine compared to all the other methyl adducts induced by MNNG.

## Studies with UV Radiation

### Excision Repair Reduces Cytotoxicity and Mutagenicity

The results of our comparative studies with normal and excision repair-deficient XP cells show that the degree of cell killing and the frequency of induction of

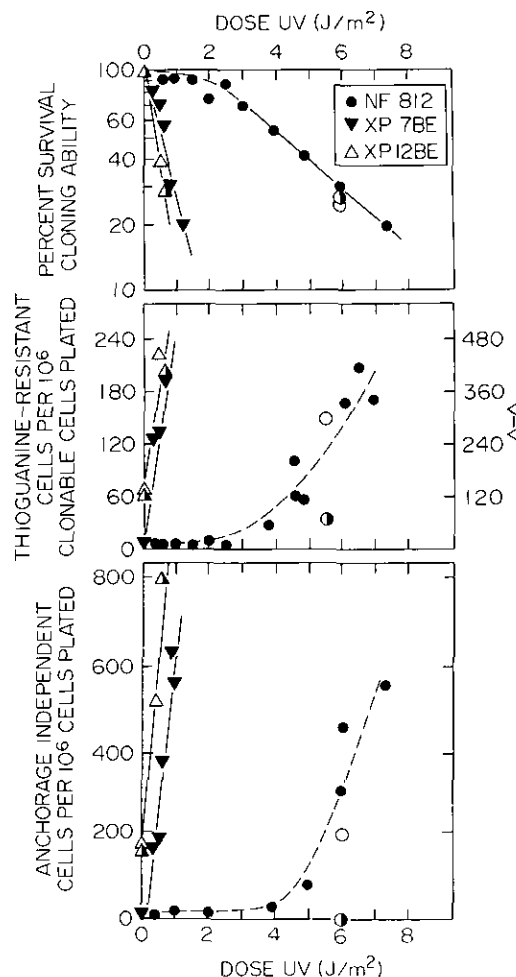


FIGURE 1. Cytotoxicity, mutagenicity, and transforming ability of UV radiation in human cells: (●, ○, ○) normal cells; (▲, Δ, Δ) XP2BE cells; (▼, ▽) XP7BE cells; (solid symbols) populations irradiated in exponential growth; (open symbols) cells synchronized by release from confluence and irradiated shortly before onset of S phase; (half-filled symbols) cells irradiated 18 to 20 hr prior to S. See text for details.

mutations to TG resistance is correlated with the cells' rate of excision of pyrimidine dimers. For example, when four cell lines, each with a cell cycle of approximately the same length but with different rates of excision of UV-induced DNA damage, were irradiated in exponential growth and assayed for survival and induction of mutations, normal cells with rapid excision were much more resistant to the potentially lethal and mutagenic effect of UV than any of the three XP strains. The XP cell line (XP12BE) with the lowest rate of nucleotide excision repair capability (29) was the most sensitive of all; those with a repair rate 16 to 20% that of normal (XP2BE, XP7BE) showed survival and mutation curves slightly more resistant than XP12BE (2,5). The normally excising human cells were the most resistant. They exhibited 100% survival and no measurable increase in mutant frequency at low doses of UV, doses that were extremely effective for the XP cells. Only when exposed to much higher doses of UV did the normal cells show a response (see Fig. 1).

Since the essential difference among these strains is their respective rates of excision repair of UV-induced damage, the data are consistent with the hypothesis that there is a certain amount of time available for excision repair between the UV irradiation and the events responsible for cell killing and mutation induction. The faster a cell can remove the potentially lethal and/or mutagenic damage from DNA, the lower the degree of cell killing and frequency of mutants. We suggested that the cellular event involved is semiconservative DNA synthesis on a damaged template (2).

### Lengthening Time between Exposure and Onset of S-Phase Decreases the Biological Effects of UV

If mutations result directly or indirectly from DNA replication on a template containing unexcised lesions, then if one were to extend the time between the introduction of the lesions and the onset of DNA synthesis, it should be possible for a cell that has at least some capacity for excision repair to remove those lesions before they can cause mutations. Similarly, if cell killing reflects replication on a damaged template, then extending the time available for excision repair before allowing cells to replicate their DNA should result in increased survival. This hypothesis was tested by growing cells to confluence to achieve density inhibition of cell replication, irradiating them, and allowing them various lengths of time to remove potentially cytotoxic or mutagenic DNA lesions. After reaching confluence, cells were refed once and then held for 72 hr without refeeding. Autoradiography studies in cells maintained in the resting state under our conditions demonstrated that < 0.5% of the cells incorporated tritiated thymidine during an 8-hr labeling period (2). However, such cells are capable of excision repair (22,24,30).

We irradiated confluent normal cells with a large enough dose of UV radiation to cause cell killing and to induce mutations in exponentially growing populations, i.e., 5 to 10 J/m<sup>2</sup>, and held them in the nonreplicating state for 7 days. XP12BE cells were given 0.5 J/m<sup>2</sup>. When the cells were trypsinized and plated at lower densities to assay survival and induction of mutations, the normal population showed no cell killing and no mutation induction. In contrast, XP12BE cells irradiated at confluence exhibited the same degree of cell killing and mutation induction as found in cycling cells (2).

To determine how much time it takes for human cells to remove potentially cytotoxic and/or mutagenic lesions induced by UV, we irradiated a series of confluent cultures and released one immediately and the others at various times following radiation. There was a gradual decrease in the lethal and mutagenic effects of the radiation. After 15 hr in the G<sub>0</sub> state, cultures of normal cells irradiated with 7 J/m<sup>2</sup>, which initially exhibited a survival of 20% and an induced mutation frequency of  $50 \times 10^{-6}$ , exhibited 100% survival and a frequency of mutations near background. XP5BE cells, with a much

slower rate of excision (19), were irradiated with only 1 J/m<sup>2</sup>, so as to obtain an initial survival of ~20%. With a 7-fold lower number of lesions to excise, these cells also showed a gradual increase in survival with time held in confluence. A period of 24 hr in the G<sub>0</sub> state was sufficient for both sets of cells to remove a sufficient number of lesions so that when they were released, they exhibited ~100% survival (2).

### Shortening Time between Exposure to DNA-Damaging Agents and Onset of S-Phase Increases the Mutagenic Effect

To see if semiconservative DNA replication of a damaged template were the cellular event responsible for cell killing and/or mutation induction, we synchronized cells by release from confluence (G<sub>0</sub> state). Autoradiography indicated that after 72 hr in a mitogen-depleted density-inhibited state, human cells plated at  $\sim 5 \times 10^3$  cells/cm<sup>2</sup> in fresh medium containing 15% fetal bovine serum begin semiconservative DNA synthesis (S-phase) after ~24 hr (31). Normal cells and XP12BE cells were irradiated under conditions that allowed 24, 18, or 1 hr to occur between irradiation and the scheduled beginning of S-phase. (The cells that were to have 24 hr before S were irradiated in confluence and then replated at lower densities. The cells that were to be irradiated 18 hr before S, or just before S, were released from confluence so as to have the appropriate number of hours prior to irradiation *in situ* at the lower densities.) The results are shown in Figure 2.

Repair-proficient normal cells irradiated shortly before S exhibited the highest frequency of mutants; those irradiated 18 to 24 hr prior to S gave a much lower frequency. XP cells showed no significant difference in mutation frequency whether irradiated in G<sub>0</sub> and released, or in early G<sub>1</sub>, or just prior to S, as predicted from their virtual lack of excision repair. These results indicate that in cells capable of removing lesions, the time available for excision repair between irradiation and S is, indeed, the determining factor for mutagenicity. If the increased mutation frequency exhibited by cells irradiated just prior to S merely reflected differences in the physical state of DNA in these cells compared with that of cells in early G<sub>1</sub>, so that a larger number of lesions were induced or lesions located in more critical sites, the XP cells would also have shown this difference.

An unexpected finding in this study was that cell killing did not show significant cell cycle dependence. When allowance is made for the shielding effect on the confluent cells and their need to be plated after irradiation, there is no significant difference in the slope of the survival curve of cells irradiated at the onset of S or 18 or 24 hr prior to S-phase (31). Yet the normally excising cells were capable of surviving 10-fold higher doses than XP12BE cells, indicating that excision repair postirradiation is involved in eliminating potentially lethal effects of UV. Based on these results and those of Kan-

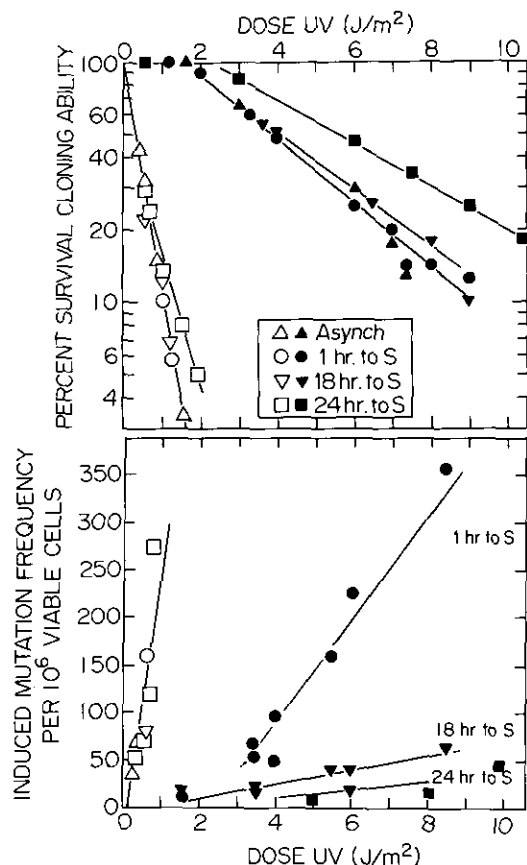


FIGURE 2. Cytotoxicity and mutagenicity of UV in ( $\blacktriangle$ ,  $\bullet$ ,  $\blacktriangledown$ ,  $\blacksquare$ ) NF cells or ( $\triangle$ ,  $\circ$ ,  $\triangledown$ ,  $\square$ ) XP12 BE cells irradiated under conditions designed to allow various lengths of time for excision repair to take place prior to the onset of S phase. Cells irradiated in confluence ( $G_0$ ) and then released and plated at lower densities ( $\blacksquare$ ,  $\square$ ); cells released from  $G_0$  and irradiated 6 hr later ( $\blacktriangledown$ ,  $\triangledown$ ); cells released and irradiated 24 hr later ( $\bullet$ ,  $\circ$ ) cells replated from asynchronously growing cultures and irradiated 16 hr later ( $\triangle$ ,  $\Delta$ ). From Konze-Thomas et al. (31) with permission.

tor et al. (32) and Hildebrand and Strniste (33), we suggested that the amount of time available for excision before cell death is regulated by the cells' need for critical cellular protein and their respective mRNAs. If the DNA template for transcription of these mRNAs is still blocked by lesions at the time the cell has need of them, reproductive death (i.e., inability to form a colony) is the result. Although the kinds of proteins being synthesized by cells in early  $G_1$  differ from those just prior to S, the overall average need for transcription and translation by the two populations may well be comparable. If so, this could explain why the lethal effect of low doses of UV was comparable in the two populations.

The hypothesis that the cells' need for some critical proteins at some point subsequent to irradiation is responsible for cell death could also explain why resting cells irradiated in confluence ( $G_0$ ) and then held for 8 or 16 hr before being allowed to cycle showed a higher survival than cells released immediately (2).  $G_0$  cells have a lower metabolic state than cells in exponential growth, and if following irradiation, fewer critical pro-

teins are needed immediately than are needed by cells released and allowed to begin the cell cycle, these cells would have time to remove the blocking DNA damage. This conclusion is consistent with the fact that XP12BE cells, which do not remove such lesions, show no dose-modifying effect when held in the  $G_0$  state (2).

## Transformation of Human Cells: Its Relationship to DNA Lesions

To see if the number of UV-induced lesions remaining unexcised correlates with induction of anchorage independence, a phenotype characteristic of tumorigenic cells, we compared irradiated normal cells, XP7BE cells and XP12BE cells for the frequency of cells able to grow in soft agar. The results are included in Figure 1 (bottom). Note that these XP cells are not malignant; the biopsies from which they are derived are always obtained from nonsunlight-exposed areas of skin.

The data in Figure 1 indicate that to achieve a particular degree of cell killing, mutagenesis, and transformation, normal cells have to be exposed to 8- to 10-fold higher doses of UV radiation than XP cells. As discussed above, this is the result expected if induction of anchorage independence, as well as TG resistance, results ultimately from DNA damage remaining unexcised in the cell at some critical time after irradiation and if, because of the difference in their respective rates of excision repair, the average number of lesions remaining at this critical time is approximately equal to the three populations.

The open symbols in Figure 1 represent data from cells irradiated shortly after the onset of S-phase; the half-open symbols are for cells treated in early  $G_1$  phase. The frequency of transformants (as well as mutants) in the normal population was much lower if time was available for excision repair prior to S-phase. As expected, this was not true of the XP12BE population. The fact that allowing substantial time for excision before DNA synthesis eliminated the potentially mutagenic and transforming effect of UV radiation in normal cells, but not in XP12BE cells, suggests that DNA synthesis on a template still containing unexcised lesion is the cellular event responsible for "fixing" the mutations and transformation. This is consistent with the findings by Kakunaga (34). He showed that when confluent cultures of a mouse cell line were exposed to 4-nitroquinoline-1-oxide and then allowed to carry out excision repair but not to replicate, the potential for focus formation was gradually eliminated. However, when the cells were allowed to undergo a single population doubling after treatment before attaining confluence, additional time in confluence did not decrease the transformation frequency.

The similarity of the dose response for the two phenotypes in Figure 1 supports the idea that acquisition of anchorage independence (transformation) in human cells occurs as the result of a single mutational event. The frequencies of mutants and transformants induced by UV differ by a factor of  $\sim 2.5$ . In our earliest experi-

ments, using propane sultone, the difference was ~22-fold (8). However, a ratio of 2.5 is much closer to the ratios we now routinely observe with a number of agents. The higher value reported in the early experiments can be attributed in part to the use of a different lot of serum and a different type of agar. Small technical differences in the way the cells were handled may also have contributed to the difference.

When cells derived from such agar colonies are isolated, propagated and assayed for ability to form tumors upon injection subcutaneously into sublethally X-irradiated athymic mice, nodules sometimes arise at the site of injection within a few weeks. These attain a maximum size of 7 to 10 mm in diameter and then stop growing and regress. Fully malignant cells derived from fibrosarcomas produce nonregressing tumors. Therefore, we suggest that acquisition of anchorage independence is one of the steps in the transformation of normal human fibroblasts and that cells with this property are partially transformed (35).

## Studies with Aromatic Amide Derivatives

These same approaches were used to determine if DNA adducts were responsible for the cytotoxic or mutagenic effect of selected chemical carcinogens. The chemicals selected were those we showed cause greater cell killing and induction of mutations in XP cells than in normal cells. Examples are the reactive derivatives of aromatic amide carcinogens, i.e., *N*-acetoxy-4-acetylaminobiphenyl (N-AcO-AABP), *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF), *N*-acetoxy-2-acetylmino-

phenanthrene (N-AcO-AAP), and *N*-acetoxy-4-acetylaminostilbene (N-AcO-AAS) (1,22,23).

Confluent cultures of normal and XP12BE cells were exposed to these four carcinogens at concentrations adjusted to result in 20% survival. One set of cells was released immediately and assayed for survival and for the number of residues bound to the DNA. The other sets were held for 18, 36, 72, or 144 hr before being released and assayed for the number of residues remaining in the DNA and for survival. The results (Fig. 3) showed that the rate of recovery was directly related to the rate of removal of the bound carcinogen residues from DNA. In the XP12BE cells, there was little or no removal of the bound material and no evidence of recovery from the potentially lethal effects of the initial exposure, even over a period of 6 days (22).

These results indicate that cell killing (loss of reproductive capacity) is directly related to the presence of unexcised adducts. They suggest that all the DNA adducts formed by a particular agent contribute to its cytotoxic effect. If only one of several adducts were cytotoxic, the rate of loss of that one would have to be proportional to the rate of loss of the total number of adducts to give the results seen in Figure 3.

Unpublished studies by Maher and McCormick comparing the mutagenic effect of N-AcO-AAF, N-AcO-AABP, and N-AcO-AAS indicate that the frequency of TG resistant mutants induced in normal cells per lethal effect (e.g., 37% survival dose) is equal. From Figure 3 it will be seen that a 37% survival dose corresponds to ca. 50 initial adducts per  $10^6$  nucleotides for N-AcO-AAF and N-AcO-AABP, but only ca. 4 adducts per  $10^6$  nucleotides for N-AcO-AAS. Additional studies with synchronized normal human cells and excision-minus

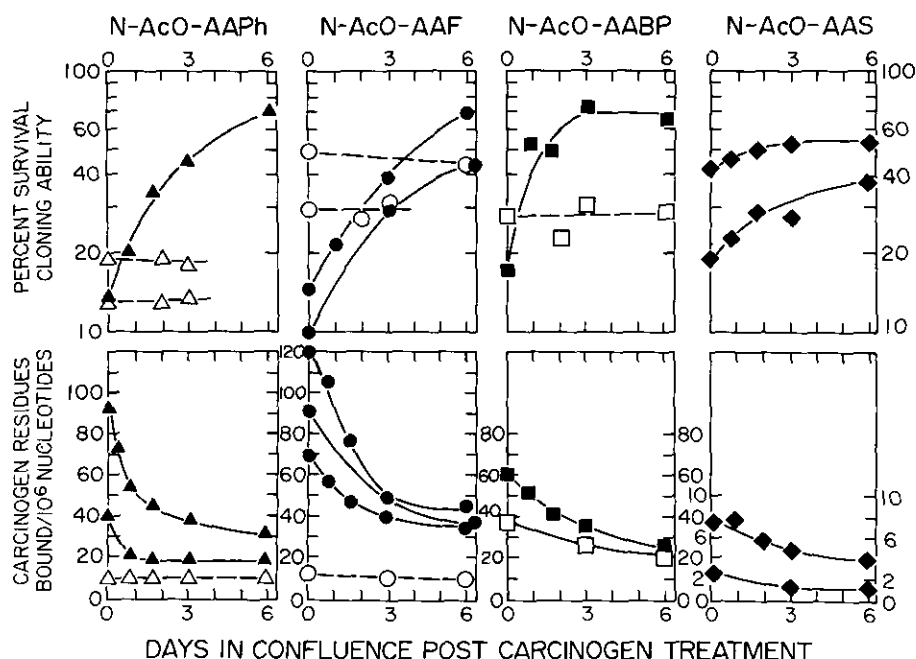


FIGURE 3. Comparison of the rate of recovery from the potentially cytotoxic effects of four aromatic amide derivatives with the rate of removal of radioactively labeled DNA adducts: (▲, ●, ■, ◆) normal cells; (△, ○, □, ◇) XP12BE cells. Cells were treated at confluence and assayed for survival after the designated period of time at confluence. From Maher et al. (23) with permission.

XP12BE cells are in progress to determine if unexcised N-AcO-AAS adducts are intrinsically more cytotoxic and mutagenic than those formed by the related derivatives, or if this observation reflects the slower rate of repair of the N-AcO-AAS-induced adducts.

## Studies with Benzo(a)pyrene-7,8-diol-9,10-epoxide

We used the same approach to investigate the kinetics of removal of DNA adducts formed by radioactive  $7\beta,8\alpha$ -dihydroxy- $9\alpha,10\alpha$ -epoxy- $7,8,9,10$ -tetrahydrobenzo(a)pyrene (anti-BPDE) and the rate of recovery of human cells from its potentially cytotoxic and mutagenic effects. Large populations of density-inhibited cells were treated, and a fraction of them were harvested immediately and assayed for DNA adducts, survival, and mutation induction. The rest were assayed after 2, 4, or 8 days in confluence (24). The results (Fig. 4) showed that residues were initially removed rapidly by the normal cells, but that after 4 days excision repair slowed considerably. Similarly, the survival of the population increased during that same period, and the frequency of mutants decreased almost to background. These data indicate that the potentially cytotoxic and mutagenic lesions were removed with about the same kinetics as were the total number of DNA adducts (24).

The HPLC characterization of the DNA adducts indicated that the major adduct was the  $N^2$ -guanyl derivative (36). Finer resolution by HPLC of these adducts, carried out by Tejwani et al. (37), indicates that at least 80% of the radioactivity is accounted for by two  $N^2$  deoxyguanosine adducts  $7\alpha$ -BPDE and  $7\beta$ -BPDE. The kinetics of decrease of tritium label in our combined  $N^2$  deoxyguanosine peak corresponded to the kinetics of decrease of radioactivity in the total DNA with time and also with the kinetics of recovery from the mutations and the cytotoxicity. If unstable adducts whose existence could not be noted, or minor adduct(s) that were not observed because of the limits of sensitivity of the assays are responsible for the mutagenic and/or cytotoxic effects of the anti-BPDE, they must be excised with the same kinetics as the  $N^2$ -guanyl derivatives to account for these results (24).

The cytotoxic and mutagenic response manifested by normal cells on release from confluence are directly related to the number of lesions in the DNA at the time the cells are plated to lower densities, regardless of whether the DNA contained this number of lesions because the cells had just been treated with anti-BPDE or because they had received a much higher dose originally and had removed some of the original adducts during the time they were held in confluence. These results indicate that RNA and protein lesions contribute only indirectly to the cytotoxic effects of such agents. Further evidence for this is shown in Figure 5. Here, the cells were exposed to 0.15, 0.2, 0.25, or 0.4  $\mu$ M tritiated anti-BPDE and assayed immediately for survival and number of adducts per  $10^6$  DNA nucleotides. The results, identified in Figure 5 with the numeral 0,

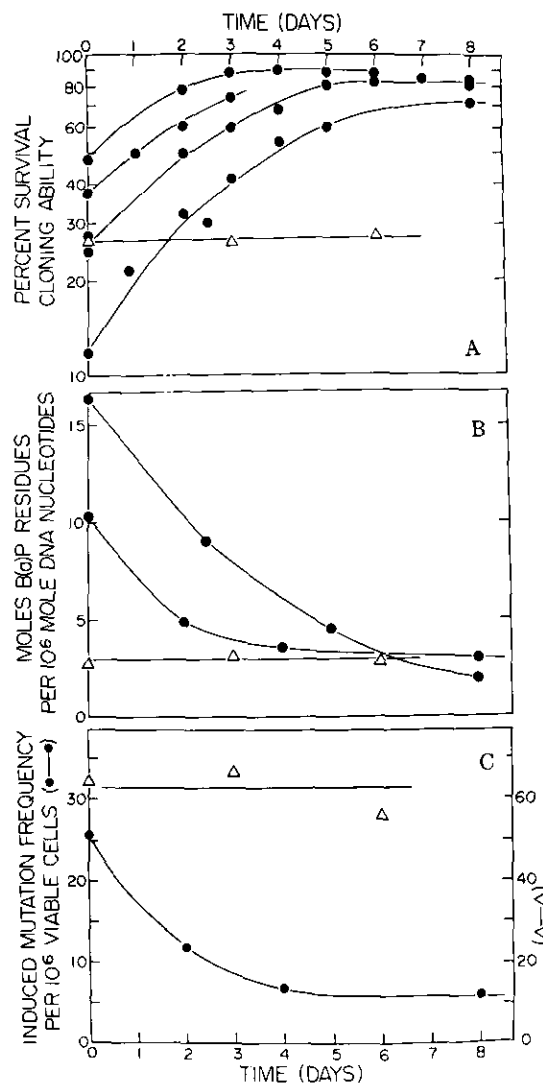


FIGURE 4. Kinetics of removal of covalently bound adducts (B) and recovery of normal cells (circles) from the potentially cytotoxic (A) or mutagenic (C) effects of anti-BPDE. The cells were treated in confluence, released on the designated days, and assayed for survival of colony-forming ability, for the number of residues bound to DNA, and after a suitable expression period, for the frequency of induced mutations to thioguanine resistance. From Yang et al (24) with permission.

show that initial binding levels of 6.5, 10.2, 14.7, and 16.3 residues per  $10^6$  nucleotides result in survivals of 40%, 27%, 16%, and 12%, respectively.

When the data from Figure 4, derived from cells treated and then held in confluence, were analyzed in the same manner and replotted with these data, the points fell on the identical line. The survival they exhibited when released from confluence was the same as that obtained with cells exposed initially to a concentration giving that number of residues per  $10^6$  nucleotides and assayed immediately. Thus, the protein binding and RNA binding that occurs with all such agents as a result of the original treatment did not contribute sig-

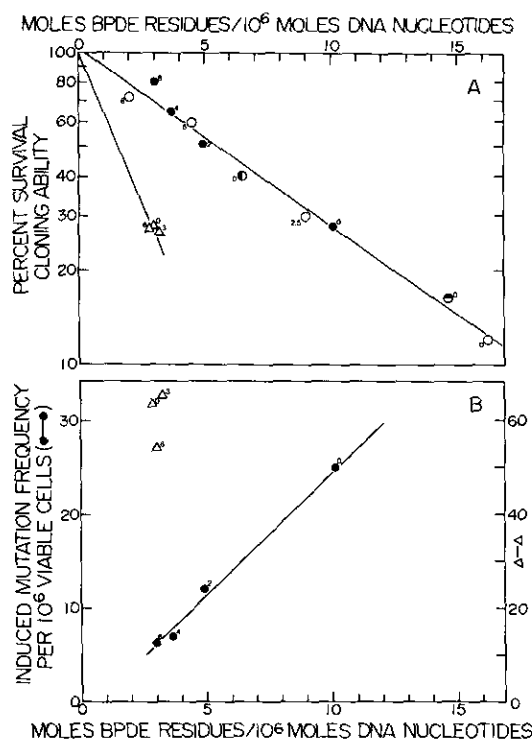


FIGURE 5. Cytotoxicity (A) and induced mutation frequency (B) as functions of the number of BPDE-induced DNA adducts present at the time cells were released from confluence and plated for survival or for the beginning of expression of thioguanine resistance: (●, ○, ●) normal cells; (Δ) XP12BE cells. The number of days cells were held in confluence before being analyzed is indicated next to each data point. The data represented by (●, ○, and Δ) have been replotted from the data given in Fig. 4. From Yang et al. (24) with permission.

nificantly to the ultimate level of cell killing. Similar conclusions can be extrapolated for mutation induction.

Additional evidence that cell killing does not reflect initial protein binding is the abnormal sensitivity of XP cells. We suggest that cell killing and mutation induction seen when cells are plated at lower densities reflect DNA lesions remaining unexcised in the cells at the time of critical events in the replicating state.

Studies by Yang et al. (25) in our laboratory with synchronized populations of normal and XP12BE cells and more recently with XP4BE by Watanabe et al. (38) gave results similar to those shown in Figure 2 for UV irradiated cells. The frequency of mutants in excision repair proficient cells is highest in cells treated with BPDE just at the beginning of S phase. Survival exhibits only a very minor cell cycle-dependence when concentrations of agents are used that do not give killing below the 10% level.

## Studies with an Aflatoxin B<sub>1</sub> Derivative

Because aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is known to be a potent liver carcinogen at comparatively low doses, we wanted to investigate its biological effects in human fibroblasts as a function of concentration applied and initial number

of DNA adducts. However, this carcinogen requires metabolic activation. Therefore, in our study (27) we substituted aflatoxin B<sub>1</sub>-dichloride (AFB<sub>1</sub>-Cl<sub>2</sub>), a direct-acting carcinogen which is a model for the proposed ultimate reactive metabolite of AFB<sub>1</sub> (the 2,3-epoxide) (39).

Figure 6 compares the cytotoxic and mutagenic effects of AFB<sub>1</sub>-Cl<sub>2</sub> as a function of the concentration administered. The slope of the survival curve for the XP12BE cells is 3-fold steeper than that of the normal cells; the slope of the induced mutation frequency curve for the XP cells is 7-fold steeper. Such differences in sensitivity might reflect differences in the amount of reactive compound entering the cells or reaching the DNA. However, this is not true for AFB<sub>1</sub>-Cl<sub>2</sub>. We found the same a linear relationship between the number of DNA adducts in either strain of cells as a function of the concentration administered. Comparison of the survival of the two strains as a function of the initial number of DNA adducts also showed the normal cells to be significantly more resistant than XP12BE cells when compared on the basis of equal amounts of DNA bound AFB<sub>1</sub>-Cl<sub>2</sub> residues. These results suggest that the normal cells are more capable than XP cells of removing potentially lethal and mutagenic lesions formed by this agent before they can exert their biological effect (27).

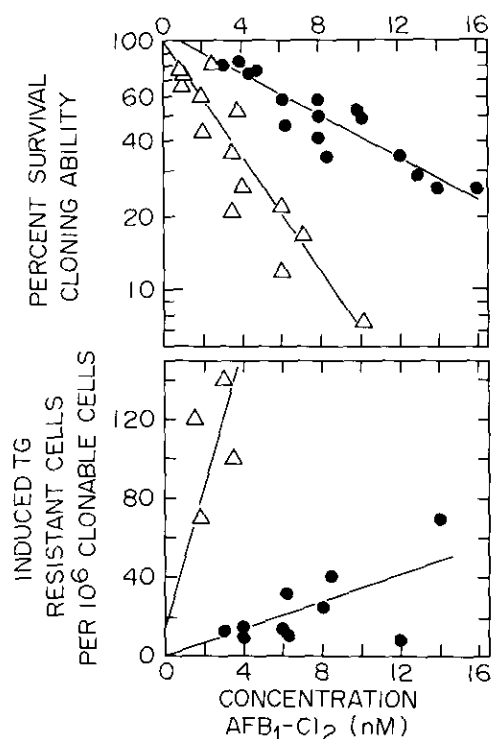


FIGURE 6. Cytotoxicity and mutagenic effect of AFB<sub>1</sub>-Cl<sub>2</sub> as a function of concentration: (Δ) XP12BE cells; (●) the normal cells. The survival was determined by treating at cloning densities or at the densities used for mutation experiments. From Mahoney et al. (27) with permission.

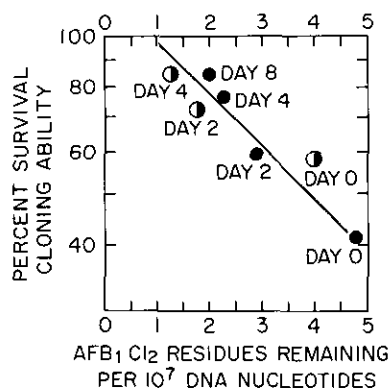


FIGURE 7. Relationship between the number of AFB<sub>1</sub>-Cl<sub>2</sub> adducts remaining bound to the DNA of normal cells harvested on the indicated days posttreatment and the corresponding cell survival. From Mahoney et al. (27) with permission.

We compared the rate of loss of AFB<sub>1</sub>-Cl<sub>2</sub>-DNA adducts from cells treated and held in the nondividing state (confluent) over several days, as well as their ability to recover from the potentially mutagenic and/or cytotoxic effect of the agent. AFB<sub>1</sub>-Cl<sub>2</sub> residues were lost from both strains of cells and both exhibited a gradual increase in survival. However, the rate of loss of adducts from the DNA in the normal cells was more rapid than in XP cells and they exhibited recovery from higher doses of AFB<sub>1</sub>-Cl<sub>2</sub> than XP cells (27).

Figure 7 shows the cytotoxicity obtained in normal cells treated in confluence and released after various periods of time as a function of the number of AFB<sub>1</sub>-Cl<sub>2</sub> residues remaining in their DNA at the time of harvest. The linear relationship obtained indicates that, at least at the concentrations of AFB<sub>1</sub>-Cl<sub>2</sub> used in our experiments, cell killing by AFB<sub>1</sub>-Cl<sub>2</sub> was directly related to the amount of unexcised DNA damage remaining in the cells at any time. The percent survival of normal cells containing 2 residues per 10<sup>7</sup> nucleotides was ~75%, whether this was the amount originally bound after a low dose, or the amount remaining after 2 days following a medium dose or after 8 days following a higher dose.

HPLC studies indicated that the major primary DNA adduct formed in the human cell and in isolated DNA was the chemically unstable guanine derivative which could undergo a change in structure with time post-treatment to form a more stable secondary adduct (27). The cytotoxic effect of AFB<sub>1</sub>-Cl<sub>2</sub> was highly correlated with the presence of either of these guanine adducts. Evidence suggests that the primary adduct is an N<sup>7</sup>-guanine adduct. The kinetics of the loss of this guanine and its transformation into the more stable secondary adduct resembled that reported recently (26) for the major primary DNA adduct formed by the reaction of AFB<sub>1</sub> at the N-7 position of guanine in the DNA of normal and XP cells and its transformation into the putative AFB<sub>1</sub>-ring opened triamino pyrimidyl structure (40).

A critical question is whether one or the other of these two major adducts is responsible for the biological effects of AFB<sub>1</sub>-Cl<sub>2</sub>. The fact that the survival of the XP

cells increased with time as the cells were held in confluence indicates that the unstable adduct was potentially cytotoxic. However, our data indicate that the stable adduct formed from the initial adduct is equally cytotoxic. This is because the level of cell killing per adduct remaining in the cells did not differ with time held in the G<sub>0</sub> state (Fig. 7), yet the nature of the adduct in the cellular DNA could be expected to change during that period. Wang and Cerutti (41) reached this same conclusion with the AFB<sub>1</sub> in 10T½ mouse embryo cells held in confluence.

Because the frequency of mutations induced by AFB<sub>1</sub>-Cl<sub>2</sub> per lethal event is relatively low, we could not apply the same technique to determine if one or both adducts are mutagenic. But the evidence shows that excision repair by normal cells reduces the potentially mutagenic effect. It may, however, do so by removing the primary lesions which would have been converted into a mutagenic secondary form. Our data emphasize that loss of AFB<sub>1</sub>-Cl<sub>2</sub> adduct posttreatment protects cells from potential mutations, i.e., repair is not responsible for the mutations.

## Studies with a Methylating Agent, MNNG

Recently, Domoradzki et al. (20) investigated the biological significance of another type of DNA repair system, i.e., O<sup>6</sup>-methylguanine-DNA-methyltransferase (MT), an acceptor protein that specifically removes methyl groups from the O<sup>6</sup> position of guanine in alkylated DNA (42). Three human cell lines were identified as being extremely deficient in this methyltransferase activity. One is an XP cell line (XP12RO) transformed to an indefinite life span by Simian virus 40 (SV40); one GM3314 is a fibroblast cell line from a skin biopsy of a patient with an inherited predisposition to colon cancer (Gardner's syndrome) and one, GM0011, is a skin fibroblast cell line from an apparently normal fetus. A fourth cell line obtained by SV 40 virus transformation of normal fibroblasts (GM637), exhibited an intermediate level of methyltransferase activity. The method of Pegg et al. (43) was used to measure the MT activity. This method uses HPLC to determine the number of O<sup>6</sup>-methylguanine bases remaining in a DNA substrate after exposure to particular amounts of protein in the cell extract.

These repair-deficient cells and a series of cell lines with normal levels of MT were compared for sensitivity to the killing and mutagenic effect of MNNG. Figure 8 (top) shows that three repair-deficient cell lines were extremely sensitive to the cytotoxic action of MNNG compared to cells with a normal ability to remove this lesion and that the response of the GM637 cells was intermediate. These data suggest that O<sup>6</sup>-methylguanine is a potentially cytotoxic lesion in human fibroblasts and that this repair system protects the cells. The data in Figure 5 (bottom) show that mutations were induced by low doses of MNNG in the three cell lines that lack the ability to remove methyl groups from the O<sup>6</sup>-position



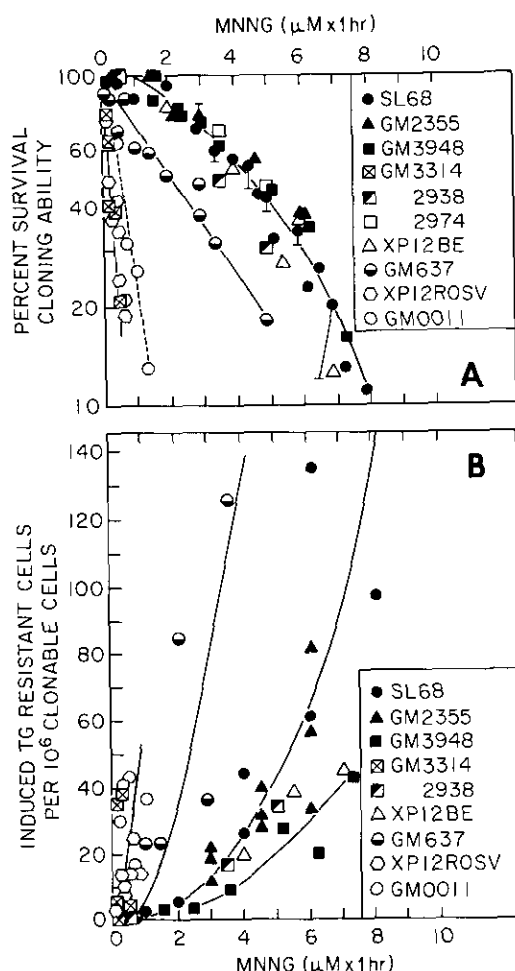


FIGURE 8. Comparison of cytotoxicity (A) and mutagenicity (B) induced by MNNG in human fibroblasts. Cells in exponential growth were treated with MNNG for 1 hr at a density of not more than  $8 \times 10^6$  cells per 150-mm dish for mutagenicity determination. See text for details. From Domoradzki et al. (20) with permission.

of guanine. There was no significant increase in the frequency of mutants in the methyl repair-proficient cells at these low doses. Only at much higher concentrations did induction of mutations occur. The GM637 cells gave an intermediate response to mutation induction.

Taken together, the biochemical and the biological data indicate that O<sup>6</sup>-methylguanine, or any other lesion that the MT can remove from methylated DNA, is the lesion responsible for mutation induction by MNNG in human cells. They also indicate that lack of MT activity is not necessarily a characteristic of skin fibroblasts of persons with a predisposition to colon cancer. This is because included in the series of normal cells shown in Figure 5 are cell lines derived from several patients with Gardner's syndrome (GM3948, 2938, 2974) and one with familial polyposis coli (GM2355). Unlike GM3314, these cell lines had a normal level of MT activity and a normal response to MNNG (20).

## Conclusion

In summary, our studies indicate that excision repair in diploid human fibroblasts is essentially an error-free process and that the ability to excise potentially cytotoxic and potentially mutagenic or transforming lesions induced in DNA by UV radiation or by several classes of chemical carcinogens determines their ultimate biologic consequences. Other classes of carcinogens that form "bulky" lesions in DNA can be expected to give similar results. Adducts produced by methylating agents are repaired by a completely different set of repair processes. The data summarized here suggest that there is a certain amount of time available between the initial exposure and the onset of the cellular events responsible for mutation induction, for cell transformation, and for cell killing and that the critical event for the mutation and transformation is DNA synthesis on a damaged template. In contrast, the cytotoxicity studies indicate that although a population's survival is determined by the extent of excision repair of potentially lethal damage from DNA before some critical cellular event(s), there is no single cell-cycle-related event such as DNA synthesis on a damaged template that determines cytotoxicity.

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